The duration of *Fgf8* isthmic organizer expression is key to patterning different tectal-isthmo-cerebellum structures

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The isthmic organizer and its key effector molecule, fibroblast growth factor 8 (Fgf8), have been cornerstones in studies of how organizing centers differentially pattern tissues. Studies have implicated different levels of Fgf8 signaling from the mid/hindbrain boundary (isthmus) as being responsible for induction of different structures within the tectal-isthmo-cerebellum region. However, the role of Fgf8 signaling for different durations in patterning tissues has not been studied. To address this, we conditionally ablated *Fgf8* in the isthmus and uncovered that prolonged expression of *Fgf8* is required for the structures found progressively closer to the isthmus to form. We found that cell death cannot be the main factor accounting for the loss of brain structures near the isthmus, and instead demonstrate that tissue transformation underlies the observed phenotypes. We suggest that the remaining Fgf8 and Fgf17 signaling in our temporal *Fgf8* conditional mutants is sufficient to ensure survival of most midbrain/hindbrain cells near the isthmus. One crucial role for sustained Fgf8 function is in repressing *Otx2* in the hindbrain, thereby allowing the isthmus and cerebellum to form. A second requirement for sustained Fgf8 signaling is to induce formation of a posterior tectum. Finally, Fgf8 is also required to maintain the borders of expression of a number of key genes involved in tectal-isthmo-cerebellum development. Thus, the duration as well as the strength of Fgf8 signaling is key to patterning of the mid/hindbrain region. By extrapolation, the length of Fgf8 expression could be crucial to Fgf8 function in other embryonic organizers.

KEY WORDS: Fibroblast growth factors, Fgf17, Inferior colliculus, Mice

INTRODUCTION

A fundamental question in developmental biology is how distinct structures are induced by a single organizing center. Since organ development occurs over time and within the changing confines of the three dimensions of space, every model must take into consideration both the length of time a cell is exposed to organizer molecules and their respective concentration. Chick transplantation studies demonstrated two decades ago that the midbrain/hindbrain boundary region (referred to as the isthmus) is a key organizing center for the midbrain as well as the cerebellum which develops from the anterior hindbrain (rhombomere 1, r1) (Joyner et al., 2000; Simeone, 2000; Nakamura, 2001; Martinez, 2001; Wurst and Bally-Cuif, 2001; Rhinn and Brand, 2001). The dorsal midbrain forms an optic tectum in fish, amphibians and birds, whereas in mammals the tectum forms an anterior superior colliculus (SC) and posterior inferior colliculus (IC) with distinct morphologies, cytoarchitecture and sensory functions (visual vs auditory processing). The cerebellum that is induced posterior to the isthmus has a homogeneous layered cytoarchitecture in most vertebrates, but a foliated structure in birds and mammals. The isthmus is a thin epithelium that links the tectum and cerebellum dorsally. The ventral midbrain and r1, by contrast, form a continuous tegmentum with distinct foci of neurons. Given the distinct structures that form in the tectal-isthmo-cerebellum region, the isthmic organizer provides an excellent model system for addressing how organizing centers function.

Fgf8 (fibroblast growth factor 8) is considered the key isthmic organizer molecule as it is both required for development of the tectum and cerebellum (Chi et al., 2003) and is sufficient to induce both structures in gain-of-function (GOF) studies (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). One aspect of the explanation for how *Fgf8* can induce different structures is likely to relate to differing strengths of intracellular signaling (Nakamura et al., 2008; Sato et al., 2004). Among the various Fgf8 protein isoforms, two are abundant in the isthmus (Blunt et al., 1997; Sato et al., 2001) and have distinct properties. Fgf8b binds the Fgf receptors (FGFRs) with higher affinity (Olsen et al., 2006), and can induce a higher level of phosphorylation of ERK than Fgf8a (Sato and Nakamura, 2004). In mis-expression studies, Fgf8a induces midbrain development, whereas Fgf8b induces cerebellum tissue (Lee et al., 1997; Liu et al., 1999; Sato et al., 2001; Liu et al., 2003). Finally, both isoforms are required, as mice lacking the four Fgf8b-like splice forms have a milder phenotype than *Fgf8*-null mice (Guo and Li, 2007).

Another consideration in studying the isthmic organizer is that two other *Fgf8* gene family members, *Fgf17* and *Fgf18*, are expressed in broader domains than *Fgf8* around the isthmus, and they have Fgf8a-like activity in gain-of-function studies (Liu et al., 2003). Since neither gene is maintained in *Fgf8* conditional knock outs (CKOs), and single mutants show mild (*Fgf17*) or no (*Fgf18*) tectum/cerebellum phenotype (Xu et al., 2000; Liu et al., 2002), their contributions to isthmic organizer activity are not clear. The secreted factor Wnt1 is also expressed near the isthmus and required for midbrain and cerebellum development, but, in GOF experiments, Wnt1 does not mimic organizer activity (Matsunaga et al., 2002; Prakash et al., 2006). Thus, Fgf8 is the only secreted factor identified to date with the full spectrum of isthmic organizer activity.

If the strength of the Fgf8 signal determines the fate of midbrain/r1 cells, then lowering the amount of *Fgf8* should result in fate changes consistent with *Fgf8*-induced structures normally found

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at a distance from the isthmus (low *Fgf8*). Indeed, unlike conditional mutants lacking *Fgf8* in the isthmus (*Fgf8$^{flox/-}$; En1$^{Cre/+}$*) that have no midbrain or r1-derived tissues (Chi et al., 2003), *Fgf8* hypomorphs (*Fgf8$^{neo/neo}$*) only develop SC and lateral cerebellum structures dorsally (Chi et al., 2003), which are tissues derived from the anterior midbrain and posterior r1, respectively (Sgaier et al., 2005). *Spry2-GOF; Fgf8$^{+/-}$* mutants that mis-express the Fgf antagonist Spry2 in the midbrain/r1 (Basson et al., 2008) and *Fgfr1$^{flox/-}$; En1$^{Cre/+}$* conditional mutants have similar phenotypes (Trokovic et al., 2003). Furthermore, in *Fgf17$^{-/-}$; Fgf8$^{+/-}$* mutants, less of the IC and cerebellum are lost (Xu et al., 2000). In *Fgf8$^{flox/-}$; En1$^{Cre/+}$* conditional mutants, massive cell death has been shown to accompany the early tissue loss; however, in *Spry2-GOF; Fgf8$^{+/-}$* and *Fgfr1$^{flox/flox}$; En1$^{Cre/+}$* mutants, this does not seem to be the case, suggesting a low level of Fgf8/17/18 signaling may be sufficient for cell survival (Chi et al., 2003; Basson et al., 2008; Trokovic et al., 2003). The results of these studies have been interpreted to mean that different levels of Fgf signaling regulate formation of different regions of the isthmo-tectal-cerebellum region, with the highest levels being required near the isthmus.

Another explanation for the mechanism of Fgf8 organizer activity would be for different parts of the tectal-isthmo-cerebellum region to require Fgf8 (and/or Fgf17/18) signaling for different lengths of time. Given that *Fgf8* is initially expressed throughout most of the dorsal r1 and the isthmus and then becomes restricted to the isthmus, it is possible that Fgf8 is only transiently required to induce posterior r1 development (lateral cerebellum). In this study, we demonstrate that more sustained *Fgf8* activity is progressively required for development of structures near the isthmus. We further show that the mechanism underlying the structural changes primarily involves Fgf8 regulating key developmental genes, rather than cell survival. Moreover, *Fgf17* and not *Fgf18* expression is maintained when *Fgf8* is temporally ablated. Thus, we have uncovered that not only the strength but also the duration of Fgf8 signaling is crucial for distinguishing the distinct structures that form along the anterior-posterior axis of the tectal-isthmo-cerebellum region in the mouse.

MATERIALS AND METHODS
Temporal inactivation of *Fgf8* in the mid/hindbrain
Fgf8$^{flox/-}$; En2$^{CreER/+}$ embryos/mice were generated by breeding *Fgf8$^{+/-}$; En2$^{CreER/CreER}$* male mice with *Fgf8$^{flox/flox}$* females. Noon on the day a vaginal plug was detected was designated as embryonic day 0.5 (E0.5). Tamoxifen (TM; Sigma T5648) was dissolved in corn oil (Sigma C-8267) at a final concentration of 20 mg/ml. 5 mg of TM per 40g of body weight was administered at noon on the days indicated. *Fgf8$^{flox/+}$; En2$^{CreER/+}$* and *Fgf8$^{+/-}$; En2$^{CreER/+}$* embryos/mice were used as controls. Genotyping was performed as previously described (Meyers et al., 1998; Sgaier et al., 2005).

Histological analysis
Embryos or E18.5 brains were immersion fixed in 4% paraformaldehyde and then embedded in paraffin and sectioned at 7 μm. Adult P21 mice were perfused and the brain fixed overnight in 4% paraformaldehyde, and then 40 μm vibratome sections were stained with Hematoxylin and Eosin.

Immunohistochemistry
Immunohistochemistry was performed on 12-14 μm frozen sections using standard procedures (see http://www.mskcc.org/mskcc/html/77387.cfm). The following primary antibodies were used: anti-neurogranin (1:500, Millipore), anti-TH (1:500, Chemicon) and anti-5-HT (1:500, Immunostar). Donkey anti-rabbit IgG-Alexa488 (1:500, Molecular Probes) or donkey anti-goat IgG-Alexa555 (1:500, Molecular Probes) were used as secondary antibodies. Brains of E18.5 embryos or P21 adults were fixed with 4% paraformaldehyde at 4°C overnight and submerged sequentially in 15% and 30% sucrose/PBS, then embedded in optimal cutting temperature (OCT) compound (Sakura).

In situ hybridization
The protocol for RNA in situ hybridization is available at the Joyner lab web site (http://www.mskcc.org/mskcc/html/77387.cfm). cDNAs for *Fgf8* (Crossley and Martin, 1995), *Fgf8* exon 3 (Lewandoski et al., 2000), *Fgf17* (Xu et al., 1999), *Fgf18* (Maruoka et al., 1998), *Spry1* (Minowada et al., 1999), *Otx2* (Ang et al., 1994) and *Wnt1* (Parr et al., 1993) were used to synthesize antisense riboprobes. Embryos were fixed in 4% paraformaldehyde/PBS, embedded in paraffin and sectioned at 6 μm. Recombination efficiency was assessed by in situ hybridization using RNA probes that detect *Fgf8* exon 3, which is deleted by Cre-mediated recombination, and compared with expression of the mutant allele using the entire *Fgf8* coding sequence (see Fig. 4).

Assay for cell death
To detect cell death, embryos were stained with LysoTrackerT (Molecular Probes L-7528) as described previously (Grieshammer et al., 2005; Basson et al., 2008).

RESULTS
Fgf8 is required during distinct temporal windows for development of different structures in the tectal-isthmo-cerebellum region
In order to uncover whether *Fgf8* is required for different lengths of time between E8.5 and E13 when it is expressed in the isthmus, we utilized an inducible Cre recombinase system to ablate *Fgf8*. We combined our *En2* knock-in allele (Sgaier et al., 2005) that expresses CreERT2 protein (Feil et al., 1996; 1997) with a conditional floxed allele of *Fgf8* (*Fgf8^{flox}*) (Meyers et al., 1998). Importantly, *En2^{CreER}* is expressed from E8.5 onwards across the mid/hindbrain junction in a domain that encompasses *Fgf8* expression in the isthmus. *Fgf8$^{flox/-}$; En2$^{CreER/+}$* mice were administered TM at E8.5, E9.5 or E10.5, and are referred to as *Fgf8-E8.5*, *-E9.5* or *-E10.5* CKOs. Two types of controls were analyzed, *Fgf8$^{flox/+}$; En2$^{CreER/+}$* littermates administered TM, and *Fgf8$^{-/+}$; En2$^{CreER/+}$* animals not administered TM (Fig. 1; also see Fig. S1M,N in the supplementary material). As CreER protein translocates into the nucleus within 6-12 hours of TM treatment, *Fgf8* function should be ablated within 24-48 hours (Danielian et al., 1998; Robinson et al., 1991; Zervas et al., 2004). Strikingly, unlike *Fgf8$^{flox/-}$; En1$^{Cre/+}$* mice that die at birth and lack the midbrain and r1, *Fgf8-E8.5*, *-E9.5* and *-E10.5* CKOs survived to adulthood. However, the mutants had a progressively greater loss of tectal-isthmo-cerebellum tissue as *Fgf8* was inactivated at earlier stages (Fig. 1; also see Fig. S1 in the supplementary material; see details below).

To determine the extent of recombination with the three TM induction time-points, we performed RNA in situ analysis with a probe that detects the *Fgf8* exon 3 sequences flanked by *loxP* sites in the conditional allele (Lewandoski et al., 2000). The three *Fgf8* temporal CKOs were analyzed at E11.5 or E12 when recombination should be complete. Analysis at this stage also allowed us to assess whether a phenotype could be detected in the mutants soon after loss of *Fgf8* function. An *Fgf8* probe containing the entire cDNA was used to assess whether the endogenous *Fgf8* gene continued to be expressed after *Fgf8* function was ablated. As previously reported (Lewandoski et al., 2000) in control embryos (*Fgf8$^{flox/+}$; En2$^{CreER/+}$*), the expression pattern detected with the exon 3 probe was the same as that with the full-length *Fgf8* probe (Fig. 2A-D). In E11.5 *Fgf8-E8.5* CKOs (3/3), no expression of *Fgf8* was detected in the dorsal isthmus with either probe (Fig. 2E-H). Furthermore, most of the dorsal isthmo-cerebellum region was deleted in these mutants, which could account for the loss of *Fgf8* expression. The phenotype nevertheless demonstrated effective knock-out of *Fgf8* in these mutants before 3 days post-TM. Consistent with the milder adult
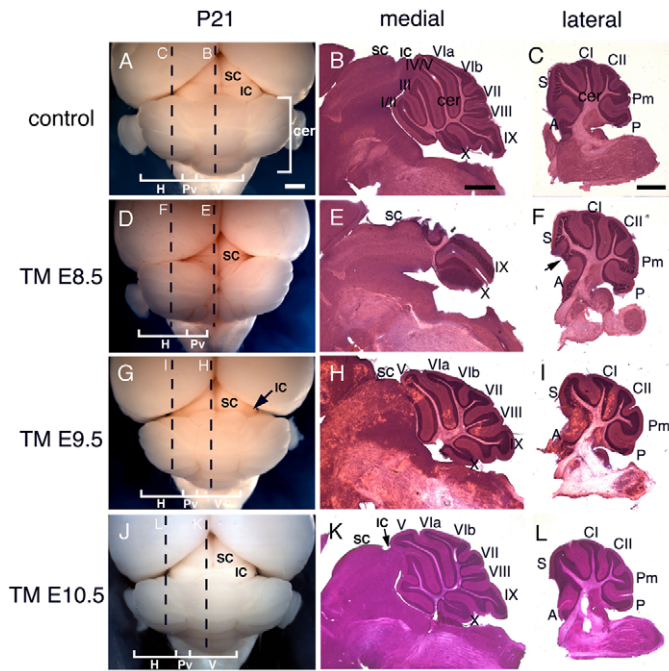
Fig. 1. Adult brain morphology shows that Fgf8 is required for different lengths of time to form tectal-isthmo-cerebellum substructures. Dorsal view of wholemount adult brains (**A**,**D**,**G**,**J**), and Hematoxylin and Eosin-stained paramedial (**B**,**E**,**H**,**K**) and lateral (**C**,**F**,**I**,**L**) sections of *Fgf8* temporal CKO embryos treated with TM at the time points indicated. The control is an *Fgf8^{flox/+}; En2^{CreER/+}* embryo treated with TM at E8.5. Arrow in F points to bulge in simplex of *Fgf8-E8.5* CKOs. Arrow in G points to loss of IC in *Fgf8-E9.5* CKOs. Arrow in K points to decreased size of the IC in *Fgf8-E10.5* CKOs. Dashed lines in wholemounts indicate the level of the indicated sections. A, anterior; cer, cerebellum; CI, crus I; CII, crus II; H, hemisphere; IC, inferior colliculus; I-X, vermis lobules, hemisphere lobules; S, simplex; SC, superior colliculus; P, copula pyramidis; Pm, paramedianus; Pv, paravermis; V, vermis. Scale bars: 1 mm.

Fig. 2. Recombination is efficient by 36 hours following TM in *Fgf8* temporal CKOs. (**A-X**) RNA in situ hybridization with the two *Fgf8* probes indicated on adjacent medial or lateral sections of *Fgf8* temporal CKO embryos treated with TM at the time-points indicated. The ages of the embryos analyzed are indicated under the arrows at the left. The control embryo is *Fgf8^{flox/+}; En2^{CreER/+}* treated with TM at E8.5. The transcripts detected with the *Fgf8* and exon 3 probes were greatly diminished in the dorsomedial isthmus (arrowheads). (K,L,O,P) Although the *Fgf8* mutant transcript was detected in the dorsolateral isthmus of *Fgf8-E9.5* CKO and *Fgf8-E10.5* CKOs, the normal transcript was greatly diminished (arrows). (E-H, asterisks) The normal *Fgf8* transcript was only partially reduced ventrally. (N,P,V,X, arrowheads) The normal *Fgf8* transcript was greatly diminished by 36 hours after TM administration in the dorsal isthmus. Scale bars: in A, 500 µm for A-P; in Q, 500 µm for Q-X.

phenotype of *Fgf8-E9.5* CKOs compared with *Fgf8-E8.5* CKOs, in three out of four E11.5 *Fgf8-E9.5* CKOs there was a reduction of tissue in the dorsal isthmus region, but less than that in *Fgf8-E8.5* CKOs (see Fig. S2 in the supplementary material for variability of recombination efficiency). Importantly, in *Fgf8-E9.5* CKOs, no expression of *Fgf8* was detected with the two *Fgf8* probes in the dorsal midline, indicating effective ablation of *Fgf8*. Faint *Fgf8* exon 3 expression was detected in some dorsal-lateral sections with the exon 3 probe and stronger expression was observed with the full length probe (Fig. 2I-L). In E12 *Fgf8-E10.5* CKOs (36 hours after TM administration), a very small number of cells were detected with the exon 3 probe, although the vast majority of cells detected with the full-length *Fgf8* probe did not express exon 3 (3/3; compare Fig. 2N,P with M,O). Moreover, the cerebellar primordium was clearly reduced in size in *Fgf8-E10.5* CKOs, but less so than in *Fgf8-E9.5* CKOs (Fig. 2M). In the ventral mid/hindbrain border region of all the *Fgf8* temporal CKOs, weak expression of *Fgf8* was detected with the exon 3 probe and stronger expression was observed with the full-length probe, especially more laterally, indicating less efficient recombination ventrally (Fig. 2E-H, asterisks).

To assess when *Fgf8* expression is extinguished in the *Fgf8* temporal CKOs, *Fgf8-E8.5* CKO embryos were assessed 24 and 36 hours after TM was administered. Expression of *Fgf8* exon 3 was
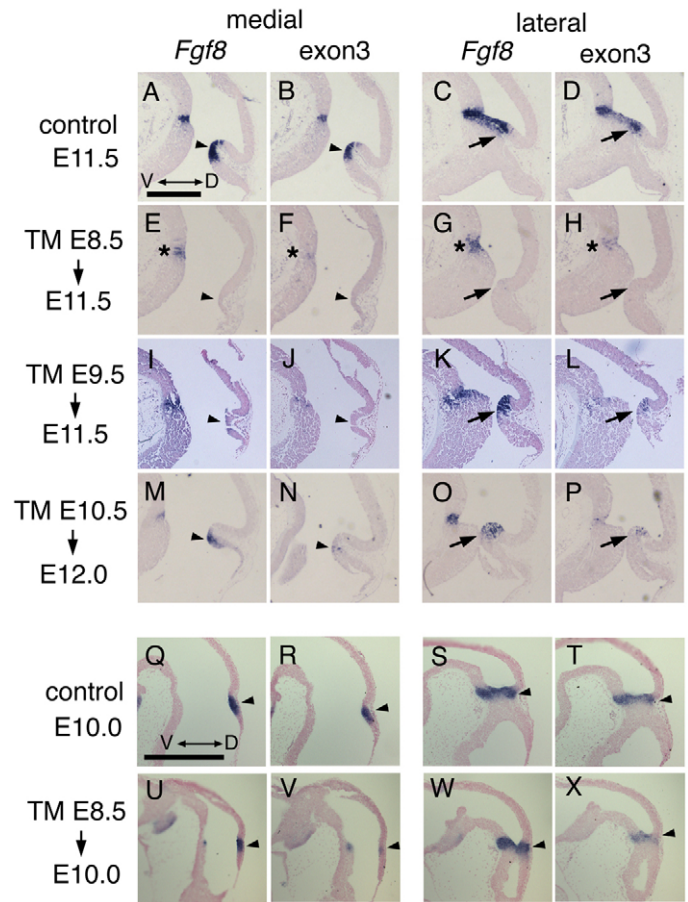
detected at 24 hours, although it appeared slightly reduced compared with controls (data not shown). By 36 hours after TM treatment, *Fgf8* exon 3 expression was greatly reduced (Fig. 2Q-X), but, as with the *Fgf8-E10.5* CKOs analyzed at E12, faint expression remained dorsally. In addition, a morphological phenotype was apparent as a reduction in the depth of the isthmus flexure (under arrowhead). Taken together, the gene expression results and timing of the onset of a phenotype (see Fig. S5 in the supplementary material, abnormal *Otx2* expression) demonstrate that our *Fgf8* temporal CKO scheme results in a decrease in *Fgf8* expression by 24 hours post-TM, with a functional decrease in Fgf8 by 36 hours. Loss of *Fgf8* is then complete in the dorsal isthmus by 48 hours and the degree of recombination is similar with the three TM treatments.
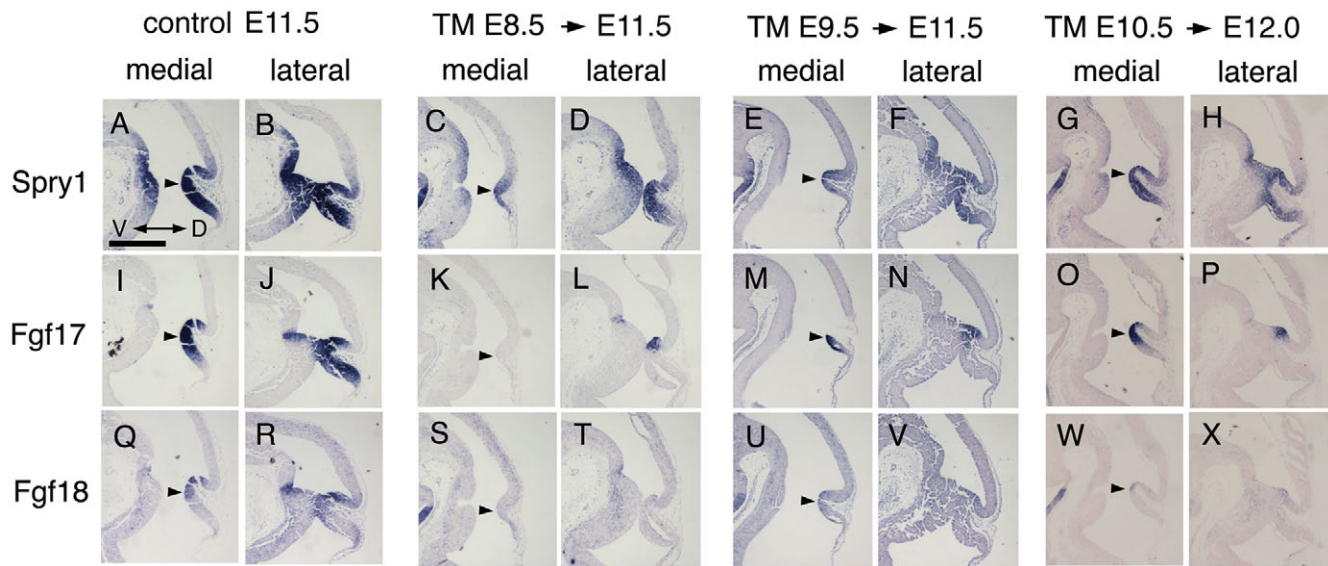
Fig. 3. Loss of *Fgf18* expression and reduction of *Fgf17* and *Sprty1* expression soon after *Fgf8* is ablated. (**A-X**) RNA in situ hybridization with the probes indicated of adjacent medial or lateral sections in each column of *Fgf8* temporal CKO embryos treated with TM at the time-points indicated. The ages of the embryos analyzed are indicated to the right of the arrows at the top. The control is an *Fgf8*^flox/+^; *En2*^CreER/+^ embryo treated with TM at E8.5. Arrowheads indicate the expressions of *Spry1* (A,C,E,G), *Fgf17* (I,K,M,O) and *Fgf18* (Q,S,U,W) in the dorsal-medial part of caudal midbrain-isthmus-r1 region. Scale bar: 500 μm.

To assess the degree to which Fgf signaling was decreased in the temporal *Fgf8* CKOs, we analyzed expression of the Fgf8 direct target gene, *Spry1* (Minowada et al., 1999; Liu et al., 2003), at E11.5 or E12 (Fig. 3). *Spry1* expression was greatly reduced in the dorsal mid/hindbrain regions of all three *Fgf8* temporal CKOs (*n*=3 for each genotype; Fig. 3A-H). The low level of Fgf signaling remaining in the mutants could be stimulated by Fgf8 released from the few remaining wild-type (WT) lateral and ventral cells and/or signaling from the related proteins Fgf17 and Fgf18 (Minowada et al., 1999). In *Fgf8* CKOs using *En1-Cre*, expression of *Fgf17* and *Fgf18* was found to be lost by approximately the 13- or 7-somite stage, respectively (Chi et al., 2003). Interestingly, although clearly reduced, *Fgf17* expression was maintained in the dorsal mid/hindbrain region of the three *Fgf8* CKOs (Fig. 3I-X), whereas *Fgf18* expression was barely detectable in all three mutants. These results demonstrate that Fgf8/17/18 signaling was greatly reduced in the three *Fgf8* temporal CKOs, but a low level of signaling remained. Importantly, based on *Spry1* expression, a similar level of signaling was present in the remaining mid/hindbrain tissue in the three *Fgf8* temporal CKO mutants, demonstrating that any differences in dorsal phenotypes in tectal-isthmo-cerebellum structures observed were due to different requirements for sustained Fgf8 signaling in specific structures.

Fgf8 is required only transiently after E8.5 for development of the superior colliculus and lateral cerebellum

We first examined the degree to which transient expression of *Fgf8* in the isthmus from E8.5 to ~E10 is sufficient to support development of tectal-isthmo-cerebellum structures by administering TM to *Fgf8*^flox/–^; *En2*^CreER/+^ embryos at E8.5, as *Fgf8* function is ablated at approximately E10. In wholemount and sections of the adult brain, *Fgf8-E8.5* CKO mice showed either a major (3/5) or partial (2/5) deletion of the medial cerebellum (vermis) compared with that of controls, as well as an absence of the inferior colliculus (IC) in all

mutants (Fig. 1A,B,D,E; also see Fig. S1A-E in the supplementary material). Based on tracing the remaining lobules on a series of sections from the vermis to the hemispheres (data not shown), and, given that only lobules VI and VII are contiguous with the four major lobules of the hemispheres and that VIII/IX extend more laterally than I-V, we suggest that the major lobules remaining in the three severe mutants correspond to lobules IX and X (Larsell, 1952). The two other mutants had a severe reduction of the anterior lobules I-V and a reduction of lobule VIII. In contrast to the medial cerebellum, the lateral cerebellum (hemispheres) appeared similar to WT, except that the simplex lobule had a small anterior bulge in all mutants (5/5; Fig. 1F, arrow). Curiously, the most anterior cerebellar tissue of all mutants was abnormally fused to the SC in the medial one-third of the mutant cerebellum (Fig. 1E, also see Fig. S1A′-E′ in the supplementary material). The tissue had a fully formed layered cytoarchitecture but the white matter (inner layer) abutted the tectum rather than the outer molecular layer (see Fig. S1 in the supplementary material, asterisks).

In order to characterize the loss of the IC further, we performed immnohistochemistry to detect the IC marker neurogranin on a series of sections along the medial-lateral axis. Whereas neurogranin marked the entire medial IC of control postnatal day 21 (P21) mice in all sections (Fig. 4B,C), in *Fgf8-E8.5* CKOs there were no neurogranin-positive cells in the medial midbrain (Fig. 4E), and only a small number of neurogranin-expressing cells were detected laterally that were abnormally positioned ventrally (Fig. 4D). These data show that the medial IC does not form and that the lateral IC develops abnormally when *Fgf8* is only transiently expressed at E8.5.

We next examined E18.5 embryonic brains (Fig. 5A-F), a stage when the isthmus is clearly visible on sections. As in the P21 *Fgf8-E8.5* CKOs, at E18.5 on medial sagittal sections of mutants, the IC was absent (Fig. 5B,E) and only a tiny cerebellum-like structure was present, fused to the caudal tip of the remaining SC (Fig. 5E, arrow). Thus, no isthmus was present in *Fgf8-E8.5* CKOs. In lateral sections, the cerebellum appeared similar to WT in morphology
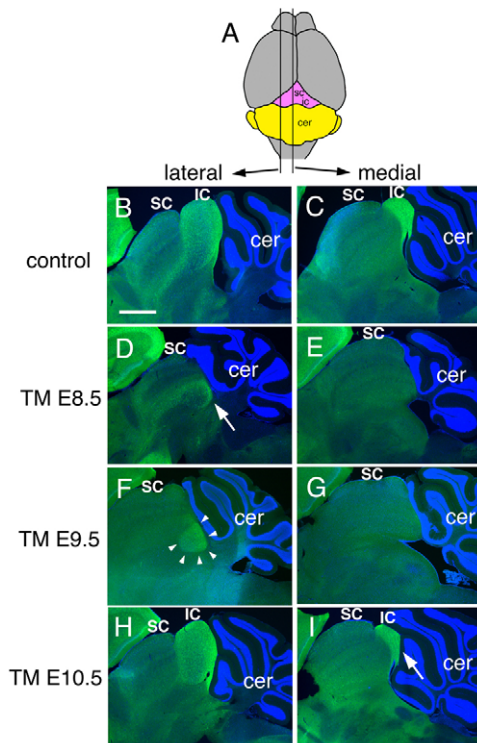
Fig. 4. Neurogranin staining of adult brain sections shows that the inferior colliculus requires sustained Fgf8 signaling. (**A**) Schematic of dorsal view of an adult brain to show the level of the lateral and medial sections shown below. (**B-I**) Neurogranin staining of the inferior colliculus of adult *Fgf8* temporal CKO embryos treated with TM at the time-points indicated. The control is an *Fgf8^{flox/+}; En2^{CreER/+}* embryo treated with TM at E8.5. The arrow in D indicates the faint and abnormally positioned neurogranin staining in the *Fgf8-E8.5* CKO. Arrowheads in F indicate the abnormal neurogranin staining in the lateral IC of a *Fgf8-E9.5* CKO mutant. Arrow in I indicates the smaller medial IC in *Fgf8-E10.5* CKO mice. Abbreviations are as in Fig. 1. Scale bar: 500 μm.

Fig. 5. E18.5 brain morphology shows that Fgf8 is required for different lengths of time to form tectal-isthmo-cerebellum substructures. Dorsal view of wholemount adult brains (**A,D,G,J**), and Hematoxylin and Eosin-stained paramedial (medial; **B,E,H,K**) and lateral (**C,F,I,L**) sections of *Fgf8* temporal CKO embryos treated with TM at the time-points indicated. The control is an *Fgf8^{flox/+}; En2^{CreER/+}* embryo. Dashed lines in wholemounts indicate the level of the indicated sections. Arrows in D and E indicate loss of the vermis, in addition to the inferior colliculus in *Fgf8-E8.5* CKO mutants, and in F the shallow isthmus flexure. The arrow in H indicates the lack of isthmus and inferior colliculus in *Fgf8-E9.5* CKO mutants, and in I the shallow isthmus flexure. The arrow in K indicates that the isthmus is missing in *Fgf8-E10.5* CKO mutants, in addition to the cerebellum fissures being shallower than normal (B,F, asterisks). Abbreviations are as in Fig. 1. Scale bars: in A, 1 mm for left column; in B, 500 μm for right two columns.

(Fig. 5C,F), whereas the midbrain was reduced in size (Fig. 5F, arrow). At E18.5 neurogranin was expressed only in the lateral IC (see Fig. S3A-C,C′ in the supplementary material). In lateral sections of *Fgf8-E8.5* CKOs, neurogranin was detected in fewer cells than normal and was abnormally positioned ventrally (see Fig. S3D-F in the supplementary material), a phenotype similar to that seen at P21.

Although recombination was less efficient ventrally, we examined the differentiation of midbrain dopaminergic neurons by assessing tyrosine hydroxylase (TH) expression and hindbrain serotonergic neurons by assessing 5-hydroxy tryptophan (5-HT) (Brodski et al., 2003; Zervas et al., 2004; Ye et al., 1998; Blaess et al., 2006). Unlike the loss of tissue in the dorsal midbrain/r1 of *Fgf8-E8.5* CKOs at E18.5, there was no obvious difference in the number of TH-positive neurons of the substantia nigra (SN) and ventral tegmental area (VTA) between control and *Fgf8-E8.5* CKO embryos (see Fig. S4A,B in the supplementary material; data not shown). However, the TH+ neurons of the locus ceruleus (LoC) in the dorsal r1 of *Fgf8-E8.5* CKO embryos had an abnormal distribution, being more spread out than normal (see Fig. S4B in the supplementary material, arrow). In only one of three mutants, there appeared to be a slight decrease in the number of 5-HT-positive neurons in the ventral r1 (see Fig. S4C,D in the supplementary material).
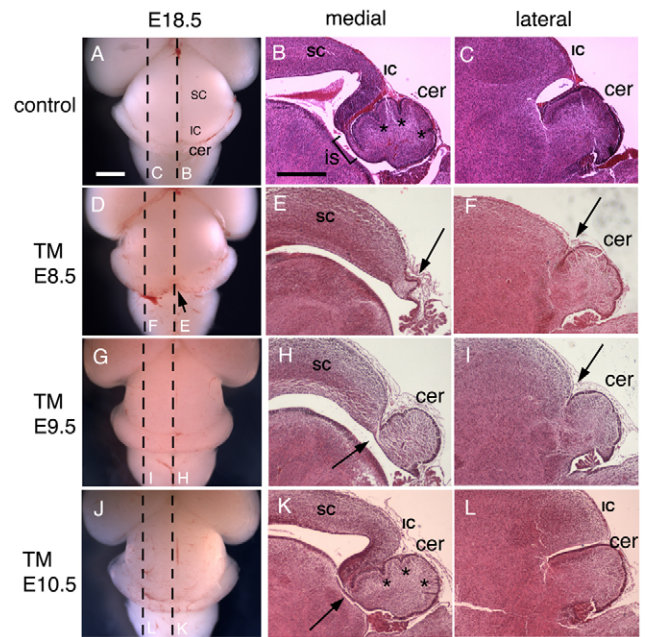
Fgf8 is required after ~E11 for development of the medial inferior colliculus and anterior-medial cerebellum

We next examined the degree to which *Fgf8* continues to be required after ~E11 for development of the vermis and IC. Strikingly, in contrast to adult *Fgf8-E8.5* CKOs, medial sections of *Fgf8-E9.5* CKO cerebellum showed that only the anterior lobules (I-V) of the vermis were missing or greatly reduced in size (*n*=4; Fig. 1H, also see Fig. S1F-I in the supplementary material). In three of the mutants, lobules I-III were replaced by cerebellum tissue that was abnormally fused to the midbrain, as in the milder *Fgf8-E8.5* CKOs (Fig. 1H; also see Fig. S1F-H in the supplementary material). In the fourth mutant, an additional small lobule was present anterior to lobule IV/V (see Fig. S1I in the supplementary material, arrow). In addition, in two mutants, the base of lobe VIII was shifted slightly posteriorly (Fig. 1H; also see Fig. S1F-I in the supplementary material). As expected, the hemispheres appeared normal (Fig. 1I). In contrast to the cerebellum, the medial IC of *Fgf8-E9.5* CKO mice was greatly reduced, based on morphology (Fig. 1G,H) and an absence of neurogranin staining in the medial sections (Fig. 4G). In the lateral midbrain, the swellings of the IC were discernible, although smaller than normal (Fig. 1G, arrow), and the domain of

neurogranin staining was shifted ventrally and greatly reduced, although less so than in *Fgf8-E8.5* CKOs (Fig. 4F). Thus, more of the vermis and lateral IC formed when *Fgf8* function was extended from ~E10 to ~E11 (difference in phenotypes obtained in *Fgf8-E8.5* CKOs compared with *Fgf8-E9.5* CKOs), but the medial IC was severely compromised.

Analysis of morphological changes in the brains of E18.5 *Fgf8-E9.5* CKOs showed defects consistent with the defects seen in adult mutants (Fig. 5G-I). The IC and isthmus were absent (Fig. 5H, arrow), and the medial cerebellum was present but was smaller than normal and possessed fewer folia. In lateral sections of *Fgf8-E9.5* CKOs, the constriction between the IC and cerebellum was more shallow than normal (Fig. 5I, arrow). Neurogranin expression was only slightly reduced compared with WTs in the most lateral tectum, whereas, on progressively more medial sections, the neurogranin staining was progressively reduced and some cells were displaced ventrally (see Fig. S3A-C,G-I in the supplementary material).

Fgf8 is required after ~E12 for development of the isthmus and to form a complete medial inferior colliculus and anterior-medial cerebellum

Finally, we examined the degree to which *Fgf8* continues to be required after ~E12 for development of the anterior vermis and medial IC. Unlike *Fgf8-E9.5* CKOs, the IC of adult and E18.5 *Fgf8-E10.5* CKOs was only slightly smaller than in controls (Fig. 1J-L, Fig. 5J-L). Neurogranin staining confirmed that the IC was present in medial and lateral sections and that the medial IC was only slightly smaller than normal (Fig. 4H,I; also see Fig. S3J-L in the supplementary material). Furthermore, only the anterior-most lobules (I-III) of the cerebellum were partially (2/3) or fully fused (1/3) and reduced in size (Fig. 1K,L; also see Fig. S1J-L in the supplementary material). By contrast, as in the other *Fgf8* temporal mutants, the most anterior region of the medial vermis contained cerebellum tissue fused to the tectum (3/3) and lobule VIII was shifted slightly posteriorly (2/3; Fig. 1K; also see Fig. S1J in the supplementary material). Furthermore, the isthmus was absent in all *Fgf8-E10.5* CKO embryos at E18.5 (Fig. 5K, arrow). As two of the *Fgf8-E10.5* CKOs had the same number of lobules as littermate controls, this suggested that the abnormal anterior cerebellum tissue fused to the tectum is ectopic tissue, possibly caused by transformation of isthmus tissue into cerebellum tissue. A much milder version of this phenotype was seen in only one control animal (0/6 *Fgf8*^+/flox^; *En2*^CreER/+^; 1/3 *Fgf8*^+/−^; *En2*^CreER/+^; see Fig. S1N in the supplementary material).

Taken together, our series of temporal *Fgf8* CKOs demonstrated that sustained Fgf8 signaling was progressively required to maintain development of structures centered around the isthmus.

The number of apoptotic cells increases only slightly in the caudal midbrain, isthmus and dorsal r1 when *Fgf8* is ablated by E10

One possible reason for the loss of tissue in the three *Fgf8* temporal CKO mutants is that cell survival was compromised. In *Fgf8*^flox/−^; *En1*^Cre/+^ CKOs, Cre is active at E8.5 and massive cell death was observed using Lysotracker (labels acidic organelles in apoptotic cells; Grieshammer et al., 2005) in the midbrain and anterior hindbrain from E8.75 to E9.50 (Chi et al., 2003). We therefore analyzed apoptosis in *Fgf8-E8.5* CKOs (the mutants with the greatest tissue loss) soon after *Fgf8* function was ablated (E10). Unlike *Fgf8* CKOs using *En1-Cre*, in the r1 of *Fgf8-E8.5* CKOs (n=6), there was only a slight increase in the number of apoptotic
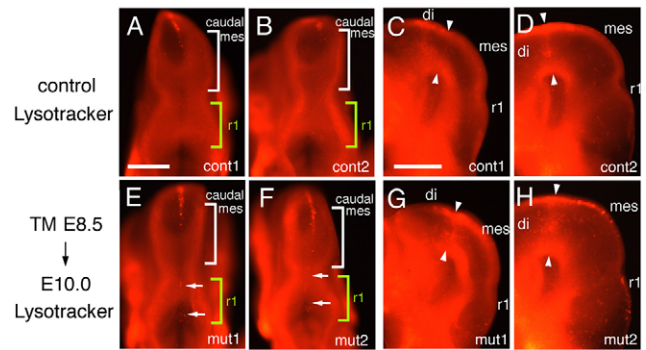

Fig. 6. Apoptosis is not greatly increased near the dorsal isthmus when *Fgf8* is ablated by ~E10. Dorsal (**A,B,E,F**) and lateral (**C,D,G,H**) views of E10 control (A-D; *Fgf8*^flox/+^; *En2*^CreER/+^, TM at E8.5) and *Fgf8-E8.5* CKO embryos (E-H) stained with Lysotracker. Arrows in E and F indicate a slight increase in apoptotic cells in the dorsal midline of the r1 in mutants. Arrowheads in G and H indicate an increase in apoptosis in the diencephalon/mesencephalic border region of mutants. di, diencephalons; mes, mesencephalon; r1, rhombomere1.

cells in the dorsal midline at E10 (Fig. 6A,B,E,F). In the midbrain of control embryos, many apoptotic cells were detected in the anterior dorsal midline (Fig. 6A,B). In E10 mutants, the dorsal midline strip of apoptotic cells extended more posteriorly than in controls (Fig. 6E,F). Curiously, in lateral regions of the anterior midbrain (primordium of the SC in normal embryos), there was an increase in the number of apoptotic cells of E10 embryos (Fig. 6C,D,G,H). To determine whether cell death is increased in *Fgf8* CKOs at a later stage, we analyzed E10.5 embryos (n=7). However, no difference in apoptosis could be detected between the CKOs and WT controls (data not shown). These results suggest that cell death is not likely to be responsible for the severe loss of the IC, isthmus and vermis in *Fgf8-E8.5* CKOs.

Otx2 and *Wnt1* are expanded posteriorly and *Gbx2* is decreased in the absence of *Fgf8* function

We next examined whether differential changes in target gene expression could account for the successively milder phenotypes seen in *Fgf8* temporal CKOs as TM was given later. The expression of *Otx2*, *Wnt1*, *Gbx2* and the *Fgf8* mutant transcript was examined, as *Otx2* and *Wnt1* are normally restricted to the midbrain and downregulated in the r1 by Fgf8 signaling, and *Gbx2* is restricted to the r1 and repressed by Otx2 (Joyner et al., 2000; Wurst and Bally-Cuif, 2001; Nakamura, 2001). Importantly, Otx2 is sufficient to transform r1 into midbrain tissue and *Gbx2* is needed to establish the presumptive r1-3 region in mice (Wassarman et al., 1997; Broccoli et al., 1999).

At E11.5 in sagittal sections of control embryos (*Fgf8*^flox/+^; *En2*^CreER/+^, TM at E8.5), *Otx2* was detected throughout the dorsal midbrain, with a posterior border at the isthmus, adjacent to the anterior border of *Fgf8* (Fig. 7A,A′,I,I′; also see Fig. S5A- E″ in the supplementary material). In lateral sections, there was a slight overlap in the expression domains of the two genes. *Wnt1* had a similar posterior border to that of *Otx2* in control embryos but was expressed only in the posterior most midbrain and in the dorsal and ventral midlines (Fig. 7E,E′; also see Fig. S5A′-E′ in the supplementary material). *Gbx2* had an anterior border adjacent to *Otx2/Wnt1* similar to *Fgf8* (Fig. 7M,M′; also see Fig. S5A‴-E‴ in the supplementary material). In medial sections of *Fgf8-E8.5* CKO embryos, *Otx2* and *Wnt1* extended to the posterior tip of what
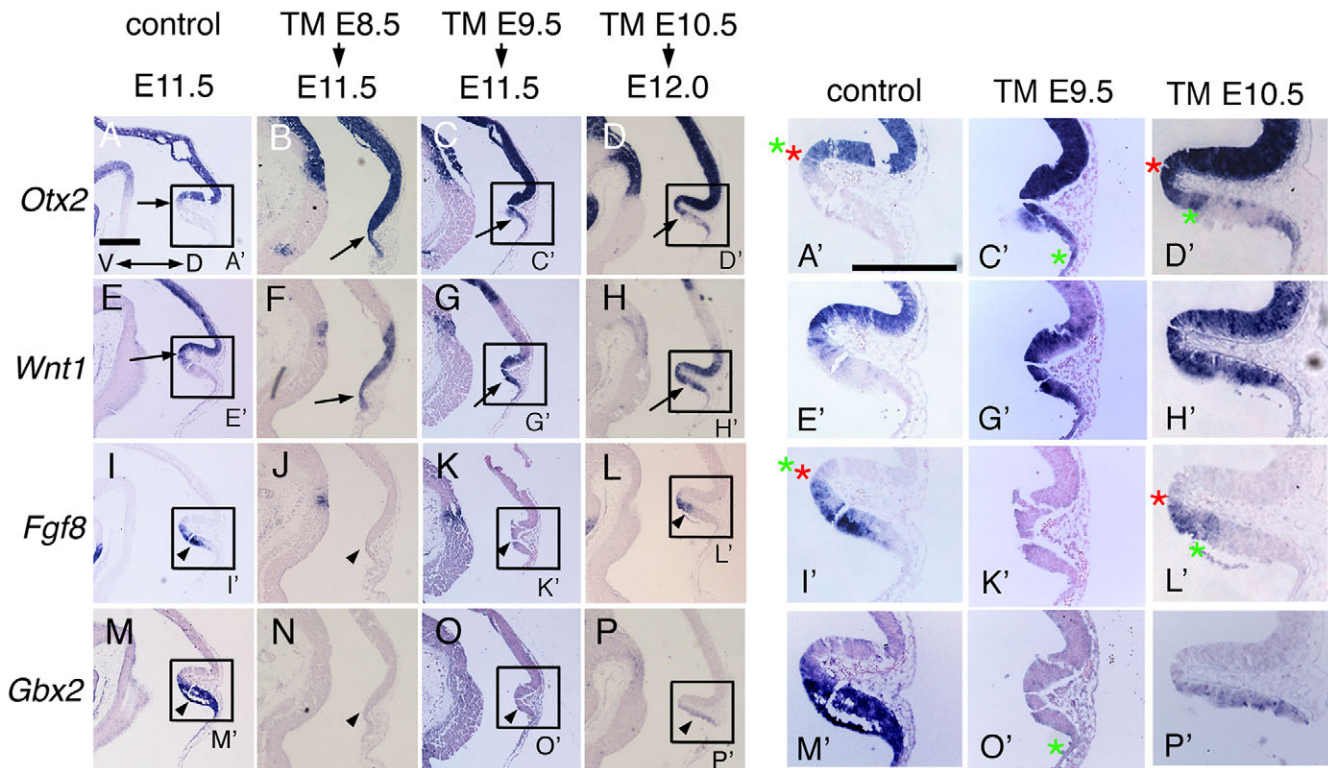
Fig. 7. *Otx2* and *Wnt1* are expanded posteriorly and *Gbx2* is diminished soon after *Fgf8* is ablated. (A-P′) RNA in situ hybridization with the probes indicated of adjacent medial sections in each column of *Fgf8* temporal CKO embryos treated with TM at the time-points indicated. The ages of the embryos analyzed are indicated under the arrows at the top. The control is an *Fgf8^{flox/+}; En2^{CreER/+}* embryo treated with TM at E8.5. Rectangles indicate area shown at higher magnification to the right. Green asterisks indicate caudal limit of the *Otx2* expression. Red asterisks indicate rostral limit of the *Fgf8* expression. Arrows in A-H indicate that *Otx2* and *Wnt1* are expanded posteriorly in the dorsal isthmus-r1 of mutants. (I-P) Dorsal *Fgf8* and *Gbx2* expression are diminished in mutants (arrowheads). Scale bar: in A, 300 μm for A-P; in A′, 300 μm for A′-P′.

remained of the tectal-isthmo-cerebellar region and *Fgf8* and *Gbx2* were absent (Fig. 7B,F,J,N; also see Fig. S5F,F′,G,G′ in the supplementary material). In more-lateral sections, *Wnt1* was expanded posteriorly to the tip of the cerebellum, whereas *Otx2* was shifted posteriorly only slightly and was absent from all but the most posterior cerebellum (see Fig. S5H-L,H′-L′ in the supplementary material). Weak expression of *Gbx2* was detected only in the lateral r1 (see Fig. S5F″-L″, lateral part of K″,L″ in the supplementary material).

In contrast to *Fgf8-E8.5* CKOs, *Otx2* was expanded posteriorly in the entire dorsal cerebellum only in the most medial sections of *Fgf8-E9.5* CKO embryos (Fig. 7C,C′; also see Fig. S5M in the supplementary material) in which no *Fgf8* mutant transcript was detected (Fig. 7K,K′; also see Fig. S5M″ in the supplementary material). In slightly lateral sections, *Otx2* was shifted only slightly posteriorly into the *Fgf8* mutant transcript domain and was absent from all but the most posterior cerebellum (see Fig. S5N-Q,N″-Q″ in the supplementary material). In *Fgf8-E9.5* CKO embryos, *Wnt1* was expanded posteriorly in the entire dorsal isthmo-cerebellum more laterally than *Otx2*, but not as laterally as in *Fgf8-E8.5* CKO embryos (Fig. 7G,G′; also see Fig. S5M′-Q′ in the supplementary material). The *Gbx2*-negative area in the isthmo-cerebellum region of *Fgf8-E9.5* CKO embryos was smaller than in *Fgf8-E8.5* CKO embryos (Fig. 7O,O′; also see Fig. S5M‴-O‴ in the supplementary material), although *Gbx2* expression appeared weaker than in controls in the lateral regions (see Fig. S5P‴,Q‴ in the supplementary material).

Correlating with the mildest phenotype in *Fgf8-E10.5* CKO embryos, *Otx2* was not expanded homogeneously to the tip of the cerebellum even in the most medial sections at E12 and instead was expanded posteriorly only into the isthmus overlapping with the *Fgf8* mutant transcript, with scattered ectopic expression in the most posterior cerebellum (Fig. 7D,D′,L,L′; also see Fig. S6A,A″,F,F″ in the supplementary material). Dorsolaterally, *Otx2* was only expanded slightly posteriorly and the amount of overlap with the *Fgf8* mutant transcript diminished progressively laterally (see Fig. S6B-E,B″-E″,G-J,G″-J″ in the supplementary material). *Wnt1* expression was expanded posteriorly to the tip of the cerebellum only in the most medial sections (Fig. 7H,H′; also see Fig. S6A′,F′ in the supplementary material), and, in more lateral sections, the caudal limit of *Wnt1* was extended only slightly posteriorly (diminishing progressively laterally) and was also expressed at the caudal tip of the cerebellum (Fig. 7H,H′; and compare Fig. S6A′-E′ with F′-J′ in the supplementary material). Finally, *Gbx2* expression was much weaker than in controls only in the most medial sections (Fig. 7P; and compare Fig. S6A‴-E‴ with F‴-J‴ in the supplementary material).

In summary, in *Fgf8* temporal CKO embryos, *Otx2* and *Wnt1* expression was expanded posteriorly into the isthmus and cerebellum, and *Gbx2* expression was correspondingly diminished. Moreover, there was a correlation between the degree to which the embryonic expression domains were altered and the degree of loss of medial cerebellum tissue in each of the adult mutants.

DISCUSSION

By conducting temporal conditional ablation of *Fgf8* in the dorsal isthmus, we have uncovered that Fgf8 signaling from the isthmus is required for different lengths of time for development of different tectal-isthmo-cerebellum structures. Importantly, the tectal-isthmo-cerebellum structures that develop from precursors increasingly further away from the isthmus at midgestation require *Fgf8* for gradually less time (Fig. 8A,C). When *Fgf8* was ablated at ~E10 (*Fgf8-E8.5* CKO), morphological and gene expression changes in the isthmus were immediately apparent. When *Fgf8* was instead ablated at ~E12 (*Fgf8-E10.5* CKO) the isthmus, anterior-medial cerebellum and part of the medial IC nevertheless did not form. Thus, sustained Fgf8 function is required to form the structures near the isthmus. Therefore, the cells that receive the highest levels of Fgf8 signaling appear to also require Fgf8 signaling for the longest duration to develop properly. Together, these findings suggest that (1) the SC and lateral cerebellum require *Fgf8* only for a brief period during E8.5-E10, (2) the posterior vermis and lateral IC require *Fgf8* during E8.5-E11, and (3) the isthmus, medial-anterior cerebellum and part of the medial IC require *Fgf8* during the entire time that *Fgf8* is expressed (E8.5-E12.5).

A key question is why the IC, isthmus and anterior-vermis are dependent on *Fgf8* for a protracted length of time. Unlike *Fgf8* CKOs using *En1-Cre* (E8.5 ablation), in which cell death appears to largely account for loss of the entire tectal-isthmo-cerebellum region (Chi et al., 2003), in *Fgf8-E8.5* CKOs (ablation at E10), only a small increase in cell death was observed near the isthmus at E10.
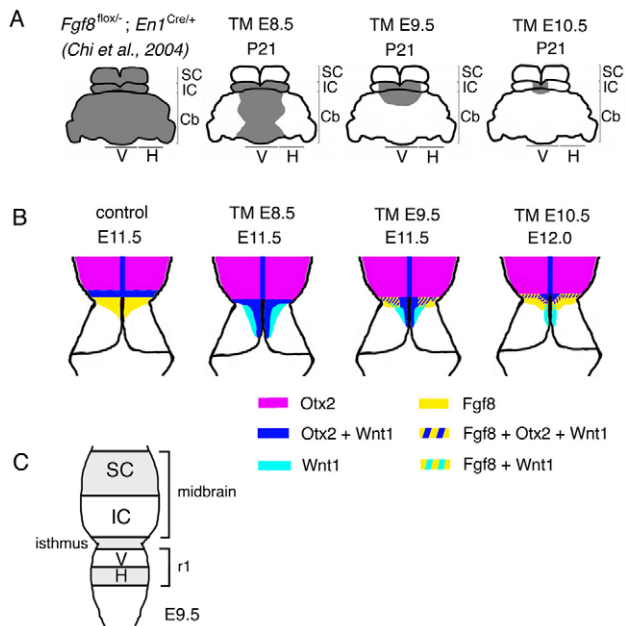

Fig. 8. Schematic showing the progressive loss of structures and more severe changes in gene expression near the isthmus as *Fgf8* is ablated earlier. (**A**) Back view of adult brain showing cerebellum [medial vermis (V) and lateral hemispheres (H)] and tectum (IC and SC). Shading indicates the regions of the tectum and cerebellum that are lost when *Fgf8* is conditionally ablated from the isthmus at the times indicated or at E8.5 in *Fgf8flox–; En1Cre* embryos (Chi et al., 2004). The isthmus is lost in all the mutants (not shown). (**B**) Changes in gene expression seen at the times indicated in *Fgf8* temporal CKO embryos. Fgf8 indicates the mutant transcript. (**C**) Dorsal view of E9.5 neural tube showing the primordial for the SC, IC, isthmus, vermis (V) and hemispheres (H).

Interestingly, in all three *Fgf8* temporal CKOs, *Fgf18* expression was lost in the dorsal mid/hindbrain region, whereas *Fgf17* expression was maintained, although reduced. We suggest that the low level of Fgf8/17 signaling that remains in the temporal *Fgf8* CKOs is sufficient to ensure survival of most midbrain/r1 cells near the isthmus. Indeed, the main increase in cell death was seen at the farthest distance from the isthmus (anterior midbrain) where Fgf signaling was not detected based on *Spry1* expression. Thus, cell death can account for only a minor part of the loss of brain structures.

If impaired cell survival does not account for loss of IC, isthmus and vermis tissue in *Fgf8* temporal CKOs, then what does? In terms of the isthmus and vermis (derived from the anterior r1), we propose that these regions are transformed into a midbrain fate owing to mis-expression of *Otx2*. Strikingly, our study showed that the degree of posterior expansion of *Otx2* into the dorsal r1 correlates with the degree of loss of the medial cerebellum in the three different temporal *Fgf8* CKOs (Fig. 8A,B). Although *Wnt1* also was expanded posteriorly in *Fgf8* temporal mutants, it is not likely to be responsible for the loss of cerebellum for several reasons. First, the mis-expression of *Otx2* correlates better with the cerebellum phenotype, as *Wnt1* was expanded much more extensively into the r1 even in *Fgf8-E10.5* CKOs that form most of the vermis. In addition, *Wnt1* mis-expression in the r1 does not cause a transformation into midbrain (Matsunaga et al., 2002; Prakash et al., 2006), whereas *Otx2* is required for tectum development (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Martinez-Barbera et al., 2001) and is capable of transforming the dorsal r1 into tectum (Millet et al., 1999; Katahira et al., 2000). The reduction in *Gbx2* expression in *Fgf8* temporal CKOs is also not likely to account for the loss of the vermis because, in *Gbx2* CKO mutants (*Gbx2flox/; En1Cre/+*), a cerebellum still develops (Li et al., 2002). Supporting the idea that mis-expression of *Otx2* in the r1 of temporal *Fgf8* CKOs is involved in the loss of medial cerebellum tissue, knock-down of *otx2* in zebrafish *fgf8* mutants (*ace*) allows differentiation of some cerebellum cell types (Foucher et al., 2006). Thus, our studies have uncovered a crucial role of sustained isthmic Fgf8 signaling in repressing *Otx2* in the isthmus and the r1, thereby creating an *Otx2*-negative region in which the isthmus and medial cerebellum can form.

The cause of the tectum phenotype seems to be distinct from that of the isthmo-cerebellum phenotype. In the temporal *Fgf8* CKOs, there was a clear increase in cell death in the anterior midbrain, unlike in the r1 where little increased apoptosis was observed. Curiously, the anterior midbrain is the primordium of the SC and a SC nevertheless forms in all three *Fgf8* temporal CKOs. A similar phenotype was seen in *Spry2-GOF; Fgf8+/–* mutants (Basson et al., 2008) where it was proposed that the cell death in the anterior midbrain was accompanied by a transformation of the remaining posterior tectum (the primordium of the IC) into SC owing to lowered Fgf signaling. We suggest that a similar mechanism underlies the *Fgf8* temporal CKO tectum phenotype. However, unlike in *Spry2-GOF; Fgf8+/–* mutants, in *Fgf8* temporal CKOs, the IC, isthmus and vermis are transformed into a SC by the remaining Fgf17 signaling and the SC precursors die owing to a lack of Fgf signaling. Thus, a second requirement for Fgf8 signaling throughout E8.5-E13 is to induce formation of a complete IC.

Our studies demonstrate a sustained requirement for Fgf8 signaling, even after E12, in maintaining the posterior gene expression borders of *Otx2* and *Wnt1*. It is possible that Fgf8 inhibits *Otx2* and *Wnt1* expression, as ectopic Fgf8b leads to their repression

(Martinez at al., 1999; Liu et al., 1999; Sato et al., 2001; Liu and Joyner, 2001; Liu et al., 2003), and reduction of Fgf8 signaling in chick embryonic r1 or in zebrafish *ace* mutants that lack only the isthmo-cerebellum region causes *otx2* to expand posteriorly (Jaszai et al., 2003; Suzuki-Hirano et al., 2005). Our study and others have found that *Gbx2* expression is reduced when Fgf8 signaling is decreased (Chi et al., 2003; Rhinn et al., 2003; Trokovic et al., 2003; Basson et al., 2008; Saarimäki-Vire et al., 2007). Since mis-expression of *Otx2* in the r1 leads to repression of *Gbx2* (Millet et al., 1999; Katahira et al., 2000), one possibility was that the ectopic Otx2 in the r1 represses *Gbx2*. However, in our *Fgf8* temporal CKOs, *Gbx2* expression was not complementary to *Otx2*. Thus, Fgf8 plays a third sustained role in maintaining the isthmus expression borders of a number of key genes involved in tectal-isthmo-cerebellum development.

Curiously, in other mouse mutants with reduced Fgf signaling that lack part of the vermis and do not have massive cell death, caudal expansion of *Otx2* into the r1 was not detected ($Fgf17^{-/-}$, $Fgf17^{-/-}$, $Fgf8^{+/-}$, $Fgf18^{-/-}$, $Fgf8^{neo/neo}$, $Fgfr1^{flox/flox}$, $En1^{Cre/+}$, $Spry2-GOF$, $Fgf8^{+/-}$) (Xu et al., 2000; Liu et al., 2002; Ohbayashi et al., 2002; Chi et al., 2003; Trokovic et al., 2003; Basson et al., 2008). Therefore, the mechanism by which the medial cerebellum is deleted in these mutants appears to be different from that of the *Fgf8* temporal CKOs. As Fgf17 (and probably Fgf18) cannot repress *Otx2* expression (Liu et al., 2003), in these mutants the remaining Fgf8 activity might be sufficient to repress *Otx2*. Basson et al. hypothesized that expansion of the roof plate in the r1 is the primary cause of loss of the vermis in *Spry2-GOF; Fgf8^{+/-}* embryos (Basson et al., 2008). As Spry inhibits pathways other than Fgf, this could account for a different mechanism in these mutants compared with that of temporal *Fgf8* CKOs. Of possible evolutionary significance, the phenotype of the *Fgf8-E8.5* CKOs most closely resembles zebrafish *ace* mutants (Jaszai et al., 2003), although the tectum is larger in the zebrafish mutants and it is not known whether an anterior transformation occurs. The results of our study thus raise the question of whether another Fgf functions early to maintain cell survival in *ace* mutants.

In summary, our study taken together with previous studies, suggest the following explanation for how a single isthmic organizer can induce distinct brain structures: during development of the dorsal tectal-isthmo-cerebellum region in mouse, the highest level and longest length of Fgf8 signaling is required for the isthmus to form. Progressively lower levels of signaling and shorter durations of signaling are required for development of tectal structures from posterior to anterior, and for regions of the cerebellum from medial to lateral (Fig. 8C). It is now important to elucidate the additional contributions of distinct Fgf8 isoforms to patterning. *Fgf8* also is required throughout E8.5 to E13 to regulate gene expression borders in the isthmus, and a sufficient level of Fgf signaling is required to maintain cell viability. Thus, the duration of *Fgf8* expression, as well as the strength of Fgf8 signaling, are both crucial factors for the specification of distinct structures within the tectal-isthmo-cerebellum region.

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